PATENT SPECIFICATION

781,584

Inventor :—JOHN LESTER SHIMWELL.

Date of filing Complete Specification : June 14, 1955.

Application Date : June 28, 1954. No. 18923/54.

Complete Specification Published : Aug. 21, 1957.

Index at Acceptance :—Class 14(2), A1.

International Classification :—C12j.

COMPLETE SPECIFICATION.

Manufacture of Vinegar.

We, BRITISH VINEGARS LIMITED, a Company organised under the laws of Great Britain, of The Vinegar Brewery, Tower Bridge Road, London, S.E.1, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement :—

The invention is an improved process of making vinegar. It includes the preparation and use of an acetic acid bacterium which, it is believed, has not hitherto been identified.

Until now the nature of the acetic acid bacteria responsible for producing the vinegar in industrial plant has remained unknown, and attempts to use pure cultures for seeding either large-scale or laboratory acetifiers have produced results inferior to those obtained with a so-called " natural " or spontaneously occurring mixed flora.

For centuries vinegar was made domestically by merely allowing " the wine of the country " to become spontaneously sour. The wine or other alcoholic beverage was allowed to remain exposed to the air, whereupon a film of acetic acid bacteria formed on the surface and acetified the alcohol beneath. This process was eventually employed commercially in France and became known as the " Orleans Process ". It is also now referred to as the " Slow Process ".

A process known as the " Quick Process " or " Trickling Process " began to become adopted in the early part of the 19th century and is now the method by which most vinegar is made all over the world. In this process the alcoholic liquor is trickled over some open-work support material such as, for example, beech wood shavings or birch twigs, whereon the acetic acid bacteria become deposited and acetify the alcohol in the liquor trickled over them. Air is passed through the support material to supply the oxygen required for the oxidation.

A more recent process, known as " The Submerged Aeration Process ", reverses the principles of the " Quick " or " Trickling " process. Instead of the alcoholic liquid being trickled in a finely divided state through a large volume of air, air in a finely divided state (bubbles) is passed through a large deep volume of the liquid, which is agitated by the stream of bubbles.

Thus, in the course of time, the manufacture of vinegar has undergone many modifications. These, however, have been almost entirely of a mechanical nature and chiefly devoted to the exploration of different methods of bringing the bacteria, the alcohol and the air into intimate contact. Little or no bacteriological progress can be noted within the vinegar industry, the bacteria actually responsible for vinegar production being chance contaminants of unknown nature. Amongst biological industries, indeed, the vinegar process enjoys the unique position of being historically one of the most ancient and bacteriologically the most backward.

There have been some attempts to isolate from mixed cultures the " true working bacteria " which are primarily if not wholly responsible for the acetification, and to apply them in practice. Thus Henneberg (*see* Handbuch der Gärungsbakteriologie, 1926, Berlin) isolated from a mixed culture employed in the " quick process " three species of *Acetobacter*, which he named *Bacterium acetigenum, Bacterium schuzenbachi* and *Bacterium curvum*. The two last-named he considered to be the chief " working bacteria " in the " quick process " and he carried out experimental acetifications with these in

laboratory acetifiers packed with beech wood shavings and wood-wool to simulate large-scale practice. Unfortunately he did not record, in the book mentioned, the volume of shavings and wood-wool relative to the volume of liquid trickled over them, so that the success or failure of the experiments is difficult to assess. It is possible, of course, to get very high acid-making rates by trickling a small quantity of liquid over a large quantity of shavings. However, Henneberg stated that in the best case 0.4—0.5% of acid was produced in 24 hours. As to the identity of *B. schuzenbachi* and *B. curvum*, Henneberg's description was not full enough to enable this to be determined today. Nor do any cultures survive in culture collections. It has, however, been pointed out that the curved form of *B. curvum* is also a characteristic of certain varieties of *A. xylinum*, notably *A. xylinum var. xylinoides* and does not exclude the possibility that the *B. curvum* of Henneberg may correspond to one of these strains.

A recent attempt at pure culture acetification is that of Lambion and Wiame (*see Bulletin Technique de la Vinaigrerie*, 1949, 4, 74). Rightly pointing out that enrichment methods are sometimes treacherous, and that direct isolation methods are often the only ones to yield the organism sought, these workers isolated a number of *Acetobacter* strains from a working acetifier. They found that strains isolated by the direct method had the desirable property of not over-oxidising the acetic acid when once it had been made, but that this occurred in the case of strains isolated by enrichment methods. They tested their best species in a laboratory acetifier with a submerged aeration process (without open work support material) and also in the same process modified by an addition of completely submerged beech shavings, and ultimately wood-wool. In the submerged aeration process without support material the acidity rose very slowly without exceeding 2.7% (w/v). With added beech shavings the acidity reached 2.7% after 25 days. With added submerged wood-wool a better result was obtained, the acidity reaching 10.5% after 25 days. These experiments were carried out using a special medium. When testing their best strain with the ordinary nutrients used in industrial spirit vinegar production they obtained a final acidity of 10.5%, after a period of 60 days.

The results of previous attempts to develop a pure culture technique in the making of vinegar have been discouraging but not destructive of the belief that there would be advantages in a process which employed a true working culture primarily if not wholly responsible for the essential acetification. The various organisms in the generally used mixed culture are obviously not all requisite for the acetification of the malt, spirit or wine liquor employed in vinegar making. Indeed, it is recognised that some of the organisms are harmful to the process, as is evident from the fact that it is customary, especially in the production of malt vinegar, to operate at a temperature high enough to inactivate undesirable bacteria and vinegar eels notwithstanding that a lower temperature would be conducive to better acetification.

The basis of the present invention is the achievement of selecting from the mixed culture normally employed in acetification a particular species of bacterium, hereinafter called *Acetobacter operans*, which is outstandingly suitable for the manufacture of vinegar under appropriate operating conditions.

A pure culture of *Acetobacter operans* is prepared by plating out in a solidifying medium acetic acid bacteria in an actively multiplying condition obtainable from a "trickling process" acetifier, the said medium serving to separate the individual bacterial cells; incubating the bacteria until visible colonies appear, and picking off from the medium such colonies of bacteria which when sub-cultured have the characteristics which are about to be stated. Preferably the original bacteria, prior to being plated out, are enriched until active multiplication of the bacteria can be detected.

Said characteristics are as follows:—

Morphology.

Variable, but usually 0.5—0.8 by 2—5μ, straight or curved. When curved, the cells are often in pairs to give a crescent shape, with the horns of the crescent noticeably thinner than the point of juncture, resembling Henneberg's drawings of *B. curvum* and to a lesser extent *B. schuzenbachi*. So-called involution forms common (in cultures of various ages) consisting of filaments with and without swellings, large swollen bodies, and spindle-shaped cells.

Catalase.—Strongly positive.

Gram-stain.—Negative.

Cultural Characteristics.

Colonies on yeast extract, 2% calcium lactate agar.—Irregularly circular, slightly convex, translucent at first becoming greyish-white. Crystals of $CaCO_3$ eventually produced.

Colonies on wort agar.—Transparent to translucent, becoming more opaque and eventually often flesh pink in colour. Slower growth than on lactate agar.

Yeast extract Ca lactate agar streak.—Translucent at first, becoming more opaque and greyish-white. Slightly raised, often glistening with moist appearance. Calcium carbonate crystals and "irisation" produced.

Wort agar streak.—Transparent, becoming translucent to opaque, often pink in colour. Texture may be similar to, or more coherent than, that of butter.

Unhopped beer gelatin streak.—Similar to wort agar, with growth adherent to medium. No softening of gelatin observed.

Unhopped beer agar streak (adjusted to *p*H 5.5).—Very good growth, often better than on calcium lactate or wort agar. Otherwise similar to latter.

Yeast extract—2% *alcohol* 2% *chalk agar streak.*—Good growth with acid production. Crystals of calcium carbonate eventually appearing, indicating over-oxidation of the acetic acid produced.

Yeast extract—10% *glucose* 2% *chalk agar streak.*—Very rapid growth with much gluconic acid produced.

Oxydogramme of calcium lactate agar.—Irisation and acetyl-methylcarbinol produced.

Liquid Media.

Hoyer's medium.—As modified by J. Frateur (*see* " La Cellule " 1950 LIII : 333).—No growth.

Unhopped beer (original gravity 1.040).—Good growth with friable delicate film, readily disintegrating and submerging on slight movement.

1% *Autolysed yeast and* 5% *alcohol* (*v*/*v*).—Similar but somewhat less abundant film than on unhopped beer.

Identification.

From the oxidation of acetic acid to CO_2 and water, and from the production of gluconic acid from glucose, this species seems to fall into the " oxydans " or " mesoxydans " groups in Frateur's classification (La Cellule, 1950, LIII, 304), which groups at present include, for example, the species A. rancens Beijerinck, *A. aceti* (Pasteur) Beijerinck, *A. xylinum* (Brown) Beijerinck, and *A. mesoxydans* Frateur.

Of the cultures which satisfy the aforesaid conditions the strains of the species *A. operans* can be readily distinguished from all the other species by their ability to produce acetic acid from a nutrient medium containing up to 7% alcohol (w/v) at a rate of not less than 20 kilograms per cubic metre of ordinary beech wood shavings per 24 hours at 30° C., and to raise the final acidity to not less than 9% acetic acid, if sufficient alcohol is provided, in the industrial manufacture of vinegar. This is in striking contrast to the rate of acetic acid production previously obtained with other *Acetobacter* species, which is recorded as being of the order of 5 kilograms of acetic acid (at the best) per cubic metre of shavings per 24 hours.

As is common knowledge in bacteriology, a " species " is not a definite entity but a taxonomic concept. Bergey's Manual of Determinative Bacteriology, 6th edition, 1948, lays this down very clearly as follows : " A species of bacterium is the type culture or specimen together with all other cultures or specimens regarded by an investigator as sufficiently like the type (or sufficiently closely related to it) to be grouped with it. It is self evident that different investigators may not draw the same boundaries for a given species." Further, there is the authority of Werner Braun in his book " Bacterial Genetics " (published by W. B. Saunders Co., Philadelphia and London, 1953) where it is stated : " Ideally, a description of a bacterial species should include its total potential mutational range." Accordingly it will be understood that the particular description hereinbefore given in respect of the species *A. operans* applies to one particular strain but that there are other strains in the species, using the term " species " in the broad sense according to the authorities quoted from as above, which are included for the purpose of the present invention notwithstanding that they do not conform with all the detailed particulars of the one strain that has been considered. It is however characteristic of all the strains of *A. operans* that they all possess the ability to produce a vinegar with a final acidity of at least 9% acetic acid at the aforesaid rate.

Features of the process, according to the invention, using *Acetobacter operans*, reside in the application of cooling to control the temperature of operation to conduce towards the optimum effectiveness of the acetification and the adaptation of the plant, including means for supplying sufficient air, for a rate of acetification which is incomparably higher than hitherto performed. In the case of the " trickling process ", to which the invention is particularly applicable, the process involves initial sterilisation of the plant, a high rate of circulation of the alcoholic liquor, and cooling of the liquor in the course of its circulation.

The invention is illustrated by the accompanying examples.

EXAMPLE 1.

Preparation of pure culture of *Acetobacter operans*.

A mixed culture of acetic acid bacteria was taken from an acetifier. Care was taken to ensure that the bacteria were in an actively multiplying condition and to this end the bacteria were first enriched by growing them in a selective acid medium consisting of 1% yeast extract, 3% acetic acid, and 4% ethyl alcohol, the medium being retained in a glass vessel, until active multiplication of the bacteria was detected. The bacteria were then plated out in solidifying media which separated the individual bacterial cells and enabled them to form visible colonies.

The solid media used were :—

(a) Beer wort agar, and (b) yeast-extract, -calcium lactate agar. Five plates of each medium were poured in serial dilution and

incubated at 30° C. until visible colonies appeared. A plate was selected on which the colonies were spaced well apart from each other and the selected colonies were picked off from this plate by means of a platinum wire and transferred to an agar slope of the same medium, and tested for high acid production.

The pure culture of *Acetobacter operans* so obtained was then grown to a sufficient volume for use in the production of vinegar. This was done by growing them in relatively shallow layers of liquid (such, for example, as yeast extract plus glucose and alcohol, or fermented malt extract) whereby a film of bacteria was grown on the surface of the liquid.

Example 2.

Manufacture of Malt Vinegar by the Trickling Process using pure culture of *Acetobacter operans*.

An acetifier employed in the production of malt vinegar was sterilised by hot water or steam and the wash used (i.e. fermented malt extract plus a small proportion of vinegar) was pasteurised to a temperature adapted to kill all organisms capable of growing therein. A pure culture of *Acetobacter operans* was then seeded on to birch twigs constituting the open work support material employed in the said acetifier. Thereafter no further seeding proved necessary, although care was taken to prevent infection from foreign organisms. The vessel employed for the acetification, of course, was resistant to attack by acetic acid and was capable of sterilisation by heat. Thus the vessel could be made of stainless steel, stoneware, or glass.

The acetification was carried out within the temperature range 75°—95° F. as opposed to the conventional temperature range of 95°—110° F. It was found that a higher rate of acetification was obtained within the temperature range 75°—95 °F. than within the said conventional temperature range.

The wash was circulated through the birch twigs at a speed such that there was a rise of temperature of only 4—6° F. between the top and bottom of the acetifier. In order to maintain this relatively small rise in temperature of the wash during its passage through the acetifier, using 2 litres of wash for every 2 litres of twigs, the wash was circulated at a rate of 30 litres per day for every 2 litres of twigs. The wash was introduced into the top of the acetifier at 82° F. and withdrawn from the bottom thereof at a temperature of 89° F., which was again reduced to 82° F. by causing the wash to flow through a heat exchange apparatus in its passage from the bottom to the top of the acetifier.

The air required for this process is very much greater than with the conventional process and it was thus forced into the acetifier, e.g. by means of a blower. The air was introduced into the top of the acetifier (although this is not essential) since the chemical reaction proceeds more vigorously at the top than at the bottom of the acetifier when the concentration of alcohol becomes low towards the end of a batch.

Although the process worked very satisfactorily with birch twigs, it was found to work even more satisfactorily with coarse wood-wool or shavings.

Whereas the amount of acetic acid produced per 24 hours from the conventional malt vinegar trickling process is in the region of 8 kilograms of acetic acid per cubic metre of birch twigs, the amount produced in the process described above is not less than 40 kilograms per cubic metre of shavings. Thus the process enables one acetifier to do the work formerly done by five acetifiers.

Example 3.

Laboratory Production of Malt Vinegar by the Trickling Process using pure culture of *Acetobacter operans*.

The process in this case was a laboratory one, the apparatus used being a tubular glass vessel of 2" bore and 5' length, which was packed with wood-wool. A malt wash was trickled over the wood-wool and fell into a bottom reservoir consisting of an aspirator from which the wash was re-circulated by means of a laboratory pump to the top of the said vessel through glass and plastic tubing.

Before acetification was started, the whole apparatus was sterilised and cooled and the wood-wool was inoculated with 200 ml. of a pure culture of *Acetobacter operans* in fermented malt extract. A mixture of 25%, pasteurised strong malt vinegar (from a works acetification) and 75% fresh filtered fermented and pasteurised malt extract was placed in the bottom reservoir. Metered air was passed in at the top of the vessel and circulation of the wash was commenced. The whole apparatus was disposed in a thermostatically controlled room, the temperature of which could be varied from room temperature up to 104° F.

Acetification started within three hours of the said inoculation with the pure culture of *Acetobacter operans*. Within 24 hours the rate of acetification was so great that the mix, which had an initial acidity of about 2.8%, was emerging from the bottom of the vessel completely converted to 7.5% vinegar. In order to slow the rate of acetification down to something of the order obtained in works practice, it was necessary to remove all but 18" of the wood-wool from the glass tube. With this depth of wood-wool and a bulk of wash in the reservoir proportional to that used in works practice for a corresponding volume of open work support material it was

found that the rate of acetification was of the order of 5.0% per day, so that a complete acetification could be carried through in some 24 hours instead of the seven or more days required on the large-scale with a mixed culture of *Acetobacter* species.

Wood-wool has a greater surface area per unit volume than have open work support material such as beech shavings, and birch twigs. When such other support materials were tried in the vessel, the result was, as expected, that the rate of acetification was roughly in proportion to the surface area of support material in the vessel. Even with support material with a surface area approximating to that used in practice, however, the rate of acetification was three to four times faster than that obtained in a works acetifier.

Example 4.

Production of Spirit Vinegar.

A laboratory acetifier was set up using new wood-wool as support material. After sterilisation and inoculation with the pure culture as described in the preceding two Examples, a spirit mix of industrial composition was circulated over the support material. The bacterial nutrient employed was compounded from yeast extract, malt extract, glucose, ammonium and potassium phosphates with magnesium and traces of other minerals, the proportions of these materials being such as is commonly used in works practice. Acetification commenced within a few hours. An acidity of 10% (w/v) was readily obtained and, on more prolonged circulation, rose to 12.0%. Thereafter, acetification was very slow. Up to an acidity of 10% the making rate over a 7 days' period averaged 20.0 to 30.0 kilogrammes of 100% acetic acid per cubic metre of support material per 24 hours.

Example 5.

Preparation of Wine Vinegar.

The process described in the preceding Example was employed with the substitution of a wine wash for a spirit wash. The wine acetified rapidly and readily and the resulting vinegar was of good bouquet and flavour.

Example 6.

Preparation of malt vinegar by the submerged aeration process.

The apparatus used for this process was a cylindrical glass vessel of 2 litre capacity, into which a total volume (inoculum and mix) of 1750 ml. was introduced. Air was supplied from a compressed air cylinder and distributed through the liquid in the form of fine bubbles by means of a sintered glass gas distributor.

Whereas in the conventional submerged aeration process there is a lag of three or more days between the inoculation of the bacteria and the start of acetification, in the present process the lag was reduced to that of only a few hours whereafter the bacteria started to grow with corresponding acid production. The average rate of acetification was of the order of 8% per day. The maximum rate of producing vinegar was not completely investigated but a production of acetic acid of 0.8% per hour was reached on several occasions. It will be appreciated that as the process starts slowly and speeds up logarithmically the average rate of acetification is lower than the maximum. The average rate was, however, so high that two complete acetifications could be carried through in one day.

As malt vinegar manufacturers carry out both the preliminary alcoholic fermentation and the subsequent acetification process themselves, the main advantage of the new process in the case of malt vinegar is the reduction in the number of acetifiers required for the manufacture of any given quantity of vinegar, and therefore the reduction of space needed to house them. In the case of spirit vinegar where the alcohol is purchased from an outside source, the advantages of the new process apply to the whole of the spirit vinegar making process as carried out by spirit vinegar manufacturers, and enables spirit vinegar to be produced more rapidly than was formerly possible.

An advantage of the new process is that a uniform product is therefore obtainable.

A pure culture of *Acetobacter operans* may be kept alive for a very long period in a suitable medium such as beer agar, by monthly sub-culture such cultures being stored at about 45° F.

What we claim is :—

1. For use in the manufacture of vinegar by acetification of alcoholic liquor, a pure culture of *Acetobacter operans* prepared by plating out in a solidifying medium acetic acid bacteria in an actively multiplying condition, obtainable for example from a trickling process acetifier, the said medium serving to separate the individual bacterial cells, incubating the bacteria until visible colonies appear, picking off from the medium such species of bacteria as have the characteristics hereinbefore defined, and selecting from said species the strains of *Acetobacter operans* distinguishable by their ability to produce acetic acid from an alcoholic nutrient medium at an outstandingly high rate.

2. A process for the manufacture of vinegar by acetification of alcoholic liquor using *Acetobacter operans* obtained in accordance with the procedure of Claim 1 whilst controlling the temperature so that it is within the range of 75—95° F., the apparatus

used, and the liquor employed, being sterilized before the commencement of operation.

3. Process according to Claim 2 in which the alcoholic liquor is circulated through support material carrying the *Acetobacter operans* at a speed such that the temperature rise between the top and bottom of the acetifier is 4—6° F.

4. Process according to Claim 2 or 3 in which the alcoholic liquor is circulated through an acetifier and is cooled during its passage from the exit from the acetifier to the inlet to the acetifier.

5. Process according to any of Claims 2 to 4 in which sufficient air is introduced into the acetifier to keep pace with a high speed of acetification which is obtained due to the use of the *Acetobacter operans*, such speed being, when using a nutrient medium containing sufficient alcohol, not less than 20 kilograms per cubic metre of ordinary beech wood shavings per 24 hours at 30° C. and to raise the final acidity to not less than 9% acetic acid.

6. Process for the manufacture of vinegar by the submerged aeration process using *Acetobacter operans* as defined in Claim 1 as the acetifying agent, the apparatus used and the liquor employed being sterilized before commencing the operation.

7. Process for producing strains of *Acetobacter operans* substantially as described in the foregoing Example 1.

8. The pure culture of a strain of *Acetobacter operans* obtained by the process of Claim 7.

9. The process for the manufacture of vinegar substantially as described in any of the foregoing Examples 2 to 6.

CARPMAELS & RANSFORD,
Agents for Applicants,
24 Southampton Buildings, Chancery Lane,
London, W.C.2.

PROVISIONAL SPECIFICATION.

Manufacture of Vinegar.

We, BRITISH VINEGARS LIMITED, a Company organised under the laws of Great Britain, of The Vinegar Brewery, Tower Bridge Road, London, S.E.1, do hereby declare this invention to be described in the following statement :—

The invention is an improved process of making vinegar. It includes the preparation and use of an acetic acid bacterium which, it is believed, has not hitherto been identified.

Until now the nature of the acetic acid bacteria responsible for producing the vinegar in industrial plant has remained unknown, and attempts to use pure cultures for seeding either large-scale or laboratory acetifiers have produced results inferior to those obtained with a so-called " natural " or spontaneously occurring mixed flora.

For centuries vinegar was made domestically by merely allowing " the wine of the country " to become spontaneously sour. The wine or other alcoholic beverage was allowed to remain exposed to the air, whereupon a film of acetic acid bacteria formed on the surface and acetified the alcohol beneath. This process was eventually employed commercially in France and became known as the " Orleans Process ". It is also now referred to as the " Slow Process ".

A process known as the " Quick Process " or " Trickling Process " began to become adopted in the early part of the 19th century and is now the method by which most vinegar is made all over the world. In this process the alcoholic liquor is trickled over some open-work support material such as, for example, beech wood shavings or birch twigs, whereon the acetic acid bacteria become deposited and acetify the alcohol in the liquor trickled over them. Air is passed through the support material to supply the oxygen required for the oxidation.

A more recent process, known as " The Submerged Aeration Process ", reverses the principles of the " Quick " or " Trickling " process. Instead of the alcoholic liquid being trickled in a finely divided state through a large volume of air, air in a finely divided state (bubbles) is passed through a large deep volume of the liquid, which is agitated by the stream of bubbles.

Thus, in the course of time, the manufacture of vinegar has undergone many modifications. These, however, have been almost entirely of a mechanical nature and chiefly devoted to the exploration of different methods of bringing the bacteria, the alcohol and the air into intimate contact. Little or no bacteriological progress can be noted within the vinegar industry, the bacteria actually responsible for vinegar production being chance contaminants of unknown nature. Amongst biological industries, indeed, the vinegar process enjoys the unique position of being historically one of the most ancient and bacteriologically the most backward.

There have been some attempts to isolate from mixed cultures the " true working bacteria " which are primarily if not wholly responsible for the acetification, and to apply them in practice. Thus Henneberg (*see* Handbuch der Gärungsbakteriologie, 1920,

Berlin) isolated from a mixed culture employed in the " quick process " three species of *Acetobacter*, which he named *Bacterium acetigenum*, *Bacterium schuzenbachi* and *Bacterium curvum*. The two last-named he considered to be the chief " working bacteria " in the " quick process " and he carried out experimental acetifications with these in laboratory acetifiers packed with beech wood shavings and wood-wool to simulate large-scale practice. Unfortunately he did not record, in the book mentioned, the volume of shavings and wood-wool relative to the volume of liquid trickled over them, so that the success or failure of the experiments is difficult to assess. It is possible, of course, to get very high acid-making rates by trickling a small quantity of liquid over a large quantity of shavings and vice versa. However, Henneberg stated that in the best case 0.4—0.5% of acid was produced in 24 hours.

As to the identity of *B. schuezenbachi* and *B. curvum*, Henneberg's description was not full enough to enable this to be determined today. Nor do any cultures survive in culture collections. It has, however, been pointed out that the curved form of *B. curvum* is also a characteristic of certain varieties of *A. xylinum*, notably *A. xylinum var. xylinoides* and does not exclude the possibility that the *B. curvum* of Henneberg may correspond to one of these strains.

A recent attempt at pure culture acetification is that of Lamboin and Wiame (*see* Bulletin Technique de la Vinaigrerie, 1949, 4, 74). Rightly pointing out that enrichment methods are sometimes treacherous, and that direct isolation methods are often the only ones to yield the organism sought, these workers isolated a number of *Acetobacter* strains from a working acetifier. They found that strains isolated by the direct method had the desirable property if not over-oxidising the acetic acid when once it had been made, but that this occurred in the case of strains isolated by enrichment methods. They tested their best species in a laboratory acetifier with a submerged aeration process (without open work support material) and also in the same process modified by an addition of completely submerged beech shavings, and ultimately wood-wool. In the submerged aeration process without support material the acidity rose very slowly without exceeding 2.7% (w/v). With added beech shavings the acidity reached 2.7% after 25 days. With added submerged wood-wool a better result was obtained, the acidity reaching 10.5% after 25 days. These experiments were carried out using a special medium. When testing their best strain with the ordinary nutrients used in industrial spirit vinegar production they obtained a final acidity of 10.5% after a period of 60 days.

The results of previous attempts to develop a pure culture technique in the making of vinegar have been discouraging but not destructive of the belief that there would be advantages in a process which employed a true working culture primarily if not wholly responsible for the essential acetification. The various organisms in the generally used mixed culture are obviously not all requisite for the acetification of the malt, spirit or wine liquor employed in vinegar making. Indeed, it is recognised that some of the organisms are harmful to the process, as is evident from the fact that it is customary, especially in the production of malt vinegar, to operate at a temperature high enough to inactivate undesirable bacteria and vinegar eels notwithstanding that a lower temperature would be conducive to better acetification.

The basis of the present invention is the achievement of selecting from the mixed culture normally employed in acetification a particular bacterium, hereinafter called *Acetobacter operans*, which is outstandingly suitable for the manufacture of vinegar under appropriate operating conditions.

A pure culture of *Acetobacter operans* is prepared by plating out in a solidifying medium acetic acid bacteria in an actively multiplying condition obtainable from a " quick process " acetifier, the said medium serving to separate the individual bacterial cells; incubating the bacteria until visible colonies appear, and picking off from the medium *Acetobacter operans*, the characteristics of which are about to be stated. Preferably the bacteria, prior to being plated out, are enriched until active multiplication of the bacteria can be detected.

Acetobacter operans may be recognized by the following characteristics :—

Morphology.

Variable, but usually 0.5—0.8 by 2—3μ, straight or curved. When curved, the cells are often in pairs to give a crescent shape, with the horns of the crescent noticeably thinner than the point of juncture, resembling Henneberg's drawings of *B. curvum* and to a lesser extent *B. schuzenbachi*. So-called involution forms common (in cultures of various ages) consisting of filaments with and without swellings, large swollen bodies, and spindle-shaped cells.

Catalase.—Strongly positive.

Gram-stain.—Negative.

Cultural Characteristics.

Colonies on yeast extract, 2% calcium lactate agar.—Irregularly circular, slightly convex, translucent at first, becoming greyish-

white. Crystals of $CaCO_3$ eventually produced.

Colonies on wort agar.—Transparent to translucent, becoming more opaque and eventually flesh pink in colour. Slower growth than on lactate agar.

Yeast extract Ca lactate agar streak.—Translucent at first, becoming more opaque and greyish-white. Slightly raised, often glistening with moist appearance. Calcium carbonate crystals and "irisation" produced. Readily detectable odour of diacetyl derived from acetylmethylcarbinol.

Wort agar streak.—Transparent, becoming translucent to opaque, pink in colour. Raised glistening growth. Texture may be similar to, or more coherent than, that of butter.

Unhopped beer gelatin streak.—Similar to wort agar, with pink growth very adherent to medium. No softening of gelatin observed.

Unhopped beer agar streak (adjusted to *p*H 5.5).—Very good growth, better than on calcium lactate or wort agar. Otherwise similar to latter.

Yeast extract—2% *alcohol* 2% *chalk agar streak.*—Good growth with acid production. Crystals of calcium carbonate eventually appearing, indicating over-oxidation of the acetic acid produced.

Yeast extract—10% *glucose* 2% *chalk agar streak.*—Very rapid growth with much gluconic acid produced. Crystals of Ca 5-ketogluconate appearing on prolonged incubation.

Yeast extract—10% *sucrose* 2% *chalk agar streak.*—Slow growth at first. After 7 days a viscous transparent growth with slow and slight production of acid.

Yeast extract—10% *maltose*, 2% *chalk agar streak.*—No growth or acid production (7 days at 27° C.).

Oxydogramme of calcium lactate agar.—Irisation and acetylmethylcarbinol produced.

Oxydogramme of 2% *glycerol agar.*—Dihydroxyacetone detected after 8 hours.

Liquid Media.

Hoyer's medium.—As modified by J. Frateur (*see* "La Cellule" 1950 LIII: 333).—No growth.

Hoyer's medium (with 1% yeast autolysate).—Heavy growth with much acid.

Unhopped beer (original gravity 40).—Good growth with friable delicate film, readily disintegrating and submerging on slight movement, liquid becoming turbid even without submergence of film; a powdery deposit eventually becoming coherent.

1% *autolysed yeast and* 5% *alcohol* (*v*/*v*).—Similar but somewhat less abundant film than on unhopped beer.

With large expanse of liquid surface, e.g. 1" depth in 1 litre flask, growth commences in the form of islands which increase in size, coalescing to form a lace-work pattern and usually eventually covering the entire surface. Such a flask culture in unhopped beer plus vinegar, containing 4% alcohol (*v*/*v*) and 3% acetic acid (*w*/*v*) (depth of liquid 1") produced 6% total acetic acid in 5 days at 30° C., whereupon over-oxidation of the acetic acid commenced.

Identification.

From the oxidation of acetic acid to CO_2 and water, from the production of gluconic acid from glucose, and from its ketogenic power, this strain falls into the "mesoxydans" group in Frateur's classification (La Cellule, 1950, LIII, 304), a group which at present comprises the species *A. aceti* (Pasteur) Beijerinck, *A. xylinum* (Brown) Beijerinck, and *A. mesoxydans* Frateur.

Features of the process, according to the invention, using *Acetobacter operans*, reside in the application of cooling to control the temperature of operation to conduce towards the optimum effectiveness of the acetification and the adaptation of the plant, including means for supplying sufficient air, to a rate of acetification which is incomparably higher than hitherto performed. In the case of the "quick process", to which the invention is particularly applicable, the process involves initial sterilisation of the plant, a high rate of circulation of the alcoholic liquor, and cooling of the liquor in the course of its circulation.

The invention is illustrated by the accompanying examples.

EXAMPLE 1.

Preparation of pure culture of *Acetobacter operans*.

A mixed culture of acetic acid bacteria was taken from a works acetifier. Care was taken to ensure that the bacteria were in an actively multiplying condition and to this end the bacteria were first enriched by suspending them in a selective acid medium consisting of 1% yeast extract, 3% acetic acid, and 4% ethyl alcohol, the medium being retained in a glass vessel. Air was supplied to the vessel in the form of fine bubbles and the supply of air was maintained until active multiplication of the bacteria was detected. The bacteria were then plated out in solidifying media which separated the individual bacterial cells and enabled them to form visible colonies.

The solid media used were:—

(a) Beer wort agar and (b) yeast-extract.-calcium lactate agar. Five plates of each medium were poured in serial dilution and incubated at 30° C. until visible colonies appeared. A plate was selected on which the colonies were spaced well apart from each other and the selected colony was picked off from this plate by means of a platinum wire and transferred to an agar slope of the same medium.

The pure culture of *Acetobacter operans* so obtained was then grown to a sufficient volume for use in the production of vinegar. This was done either by submerged aeration with bubbles of air in a relatively deep volume of liquid or statically in relatively shallow layers of liquid (such, for example, as yeast extract plus glucose and alcohol, or fermented malt extract) whereby a film of bacteria was grown on the surface of the liquid. Both methods of producing a sufficient volume of *Acetobacter operans* culture were found equally efficacious.

Example II.

Manufacture of Malt Vinegar by the Trickling Process using pure culture of *Acetobacter operans*.

An acetifier employed in the production of malt vinegar was sterilised by hot water or steam and the wash used (i.e. fermented malt extract plus a small proportion of vinegar) was pasteurised to a temperature adapted to kill all organisms capable of growing therein. A pure culture of *Acetobacter operans* was then seeded on to birch twigs constituting the open work support material employed in the said acetifier. Thereafter no further seeding proved necessary, although care was taken to prevent infection from foreign organisms. The vessel employed for the acetification, of course, was resistant to attack by acetic acid and was capable of sterilisation by heat. Thus the vessel could be made of stainless steel, stoneware, or glass.

The acetification was carried out within the temperature range 75—95° F. as opposed to the conventional temperature range of 95—110° F. It was found that a higher rate of acetification was obtained within the temperature range 75—95° F. than within the said conventional temperature range.

The wash was circulated through the birch twigs at a speed such that there was a rise of temperature of only 4—6° F. between the top and bottom of the acetifier. In order to maintain this relatively small rise in temperature of the wash during its passage through the acetifier, using 2 litres of wash for every 2 litres of twigs, the wash was circulated at a rate of 30 litres per day. The wash was introduced into the top of the acetifier at 82° F. and withdrawn from the bottom thereof at a temperature of 86° F., which was again reduced to 82° F. by causing the wash to flow through a heat exchange apparatus in its passage from the bottom to the top of the acetifier.

The air required for this process is very much greater than with the conventional process and it was thus forced into the acetifier, e.g. by means of a blower. The air was introduced into the top of the acetifier since the chemical reaction proceeds more vigorously at the top than at the bottom of the acetifier when the concentration of alcohol becomes low towards the end of a batch.

Although the process worked very satisfactorily with birch twigs, it was found to work even more satisfactorily with wood-wool.

Whereas the amount of acetic acid produced per 24 hours from the conventional malt vinegar trickling process is in the region of 8 kilograms of acetic acid per cubic metre of birch twigs, the amount produced in the process described above is not less than 40 kilgrams per cubic metre of twigs. Thus the process enables one acetifier to do the work formerly done by five acetifiers.

Example III.

Laboratory Production of Malt Vinegar by the Trickling Process using pure culture of *Acetobacter operans*.

The process in this case was a laboratory one, tho apparatus used being a tubular glass vessel of 2" bore and 5' length, which was packed with wood-wool. A malt wash was trickled over the wood-wool and fell into a bottom reservoir consisting of an aspirator from which the wash was re-circulated by means of a laboratory pump to the top of the said vessel through glass and plastic tubing.

Before acetification was started, the whole apparatus was sterilized and cooled and the wood-wool was inoculated with 200 ml. of a pure culture of *Acetobacter operans* in fermented malt extract. A mixture of 25% pasteurized strong malt vinegar (from a works acetification) and 75% fresh filtered fermented and pasteurized malt extract was placed in tho bottom reservoir. Metered air was passed in at the bottom of the vessel and circulation of the wash was commenced. The whole apparatus was disposed in a thermostatically controlled room, the temperature of which could be varied from room temperature up to 100° F.

Acetification started within three hours of the said inoculation with the pure culture of *Acetobacter operans*. Within 24 hours the rate of acetification was so great that the mix, which had an initial acidity of about 2.8% was emerging from the bottom of the vessel completely converted to 7.5% vinegar. In order to slow the rate of acetification down to something of the order obtained in works practice, it was necessary to remove all but 18" of the wood-wool from the glass tube. With this depth of wood-wool and a bulk of wash in the reservoir proportional to that used in works practice for a corresponding volume of open work support material, it was found that the rate of acetification was of the order of 5.0% per day, so that a complete acetification could be carried through in

some 24 hours instead of the seven or more days required on the large-scale with a mixed culture of *Acetobacter* species.

Wood-wool has a greater surface area per unit volume than have open work support materials such as beech shavings, and birch twigs. When such other support materials were tried in the vessel, the result was, as expected, that the rate of acetification was roughly in proportion to the surface area of support material in the vessel. Even with support material with a surface area approximating to that used in practice, however, the rate of acetification was three to four times faster than that obtained in a works acetifier.

EXAMPLE IV.

Production of Spirit Vinegar.

A laboratory acetifier was set up using new wood-wool as support material. After sterilization and inoculation with the pure culture as described in the preceding two examples, a spirit mix of industrial composition was circulated over the support material. The bacterial nutrient employed was compounded from yeast extract, malt extract, glucose, ammonium and potassium phosphates with magnesium and traces of other minerals, the proportions of these materials being such as is commonly used in works practice. Acetification commenced within a few hours. At first the vinegar which left the support material at the bottom of the acetifier was slightly turbid with bacteria, but this soon cleared up and for all further acetifications a perfectly clear effluent vinegar was obtained. An acidity of 10% (w/v) was readily obtained and, on more prolonged circulation, rose to 12.0%. Thereafter acetification was very slow. Up to an acidity of 10% the making rate over a 7 days' period averaged 20.0 to 30.0 kilogrammes of 100% acetic acid per cubic metre of support material per 25 hours.

EXAMPLE V.

Preparation of Wine Vinegar.

The process described in the preceding example was employed with the substitution of a wine wash for a spirit wash. The wine acetified rapidly and readily and the resulting vinegar was of good bouquet and flavour.

EXAMPLE VI.

Preparation of malt vinegar by the submerged aeration process.

The apparatus used for this process was a cylindrical glass vessel of 2 litre capacity, into which a total volume (inoculum and mix) of 1750 ml. was introduced. Air was supplied from a compressed air cylinder and distributed through the liquid in the form of fine bubbles by means of a sintered glass gas distributor.

Whereas in the conventional submerged aeration process there is a lag of three or more days between the inoculation of the bacteria and the start of acetification, in the present process the lag was reduced to that of only a few hours whereafter the bacteria started to grow with corresponding acid production. The average rate of acetification was of the order of 8% per day. The maximum rate of producing vinegar was not completely investigated but a production of acetic acid of 1.0% per hour was reached on several occasions. It will be appreciated that as the process starts slowly and speeds up logarithmically the average rate of acetification is lower than the maximum. The average rate was, however, so high that two complete acetifications could be carried through in one day.

As malt vinegar manufacturers carry out both the preliminary alcoholic fermentation and the subsequent acetification process themselves, the main advantage of the new process in the case of malt vinegar is the reduction in the number of acetifiers required for the manufacture of any given quantity of vinegar, and therefore the reduction of space needed to house them. In the case of spirit vinegar where the alcohol is purchased from an outside source, the advantages of the new process apply to the whole of the spirit vinegar making process as carried out by spirit vinegar manufacturers, and enables spirit vinegar to be produced more rapidly than was formerly possible.

An advantage of the new process is that acetification occurs at an even rate and a uniform product is therefore obtainable.

A pure culture of *Acetobacter aperans* is not contaminated by foreign organisms during the vinegar making and may be kept alive for a very long period in a suitable medium such as agar.

CARPMAELS & RANSFORD,
Agents for Applicants,
24 Southampton Buildings, Chancery Lane,
London, W.C.2.

Abingdon: Printed for Her Majesty's Stationery Office, by Burgess & Son (Abingdon), Ltd.—1957.
Published at The Patent Office, 25, Southampton Buildings, London, W.C.2,
from which copies may be obtained.